Rate of Chondroitin Sulfate Formation in Wound Healing

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THE ROLE of mucopolysaccharides in the formation of connective tissue components, notably collagen, has been of interest for many years, particularly among workers concerned with problems of wound healing.

One approach to the elucidation of this role has been measurement of histologic and chemical changes in wound granulation tissue as healing progresses. Dunphy and Udupa [8] determined the content of hydroxyproline as a measure of collagen, and the content of hexosamine as a measure of mucopolysaccharides in open skin wounds of rats between three and fifteen days after wounding. A variety of staining procedures was carried out on sections of tissue in an attempt to visualize changes in the collagen and mucopolysaccharide components. On the bases of carbohydrate staining procedures (toluidine blue,[11] colloidal iron [18] and periodic acid Schiff [13]) and hexosamine content it was proposed that the early *substrate phase* of wound healing is characterized by production of acid mucopolysaccharides and soluble precursors of collagen. These components rose to maximum at four to six days following wounding and declined thereafter. Collagen fibers appeared to be formed later. Similar conclusions had been reached earlier by Balazs and Holmgren [3] who measured the binding of toluidine blue by saline extracts and showed that extracts of recent wounds exhibited more dye binding than those of earlier wounds. Therefore, it was possible that synthesis of a mucopolysaccharide rich-ground substance was necessary for subsequent collagen formation.

Toluidine blue metachromasia is not a good measure of the amount of mucopolysaccharide in tissue since it is not absolutely specific and it cannot be considered quantitative. Total tissue hexosamine is a particularly poor criterion of mucopolysaccharide content since sundry other substances contain hexosamine. Indeed, in a later study Edwards *et al.*[9] found a high concentration of hexosamine six hours after subcutaneous implantation of polyvinyl sponges, a finding which pointed to plasma glycoproteins as the source of hexosamine.

The first attempt to isolate mucopolysaccharides from granulation tissue was made by Jackson *et al.*[14] who showed a fairly constant concentration of chondroitin sulfate in wound tissue between days six and fifteen. This concentration was extremely low and did not permit adequate characterization of the polysaccharide.

Improvements in procedures for isolation of tissue mucopolysaccharides permitted a more detailed study of carbohydrates of wound tissue and it was found that chondroitin-4-sulfate (CSA-A) and dermatan sulfate (CSA-B) together with relatively small amounts of hyaluronic acid were the only mucopolysaccharides present in granulation tissue ten days following wounding.[4]

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The present study attempts to demonstrate changes occurring in the concentrations of these components during healing.

Methods

Dorsal wounds were inflicted upon 60 male Sprague-Dawley rats weighing between 200 and 250 Gm. Circular pieces of skin, 2 inches in diameter, were removed under light ether anesthesia. Five rats were decapitated on alternate days from 3 to 21 days following wounding and the granulation tissue was dissected and weighed. Insufficient tissue (273 mg.) was obtained from 3-day-old wounds. The scab was discarded and histologic examination showed that insignificant amounts of granulation tissue were lost.

Tissue Treatment. Tissue was homogenized in a Virtis homogenizer at 45,000 rpm in phosphate buffer pH 8; 5 ml. buffer/Gm. granulation tissue. Following homogenization, tissue was digested with 2× crystallized papain as described by Antonopoulos *et al.*[2] at 65° C. for 8 hours. The mixture was clarified by high speed centrifugation and the supernatant dialyzed salt free. Mucopolysaccharide was precipitated from the clear dialysate by the addition of ethanol to a concentration of 70% together with trace amounts of sodium acetate. The precipitate was redissolved in a small amount of water and reprecipitated by the addition of a small amount of cetyl pyridinium chloride (CPC).[19] The CPC-MPS precipitate was redissolved in 60% propanol and again precipitated by the addition of ethanol and sodium acetate. This precipitate was washed in absolute ethanol, dry ether, and dried *in vacuo*.

It is recognized that papain treatment will alter drastically the protein-polysaccharide complex existing in tissue, but for the purposes of this investigation, in which quantitation of carbohydrate moiety is the major object, this treatment was believed justifiable.

Mucopolysaccharide Separation. Separation of mucopolysaccharide-cetyl pyridinium chloride complexes was achieved by the cellulose micro-column technique of Antonopoulous *et al.*[1] as previously described.[4] Eight columns were run on the mucopolysaccharides isolated from each time point. The solvents used were of the same concentrations as those previously described, i.e., 1% CPC, 0.3M NaCl, 0.75M $MgCl_2$ in 0.1% acetic acid, 0.75M $MgCl_2$ in water and 2M $MgCl_2$; all containing 0.05% CPC. It was assumed that material isolated with these solvents would be identical to that isolated earlier[4] from the same tissue (*vide infra*).

Analytic Procedure. The material isolated with 0.3M NaCl and with 1% CPC was hydrolyzed directly by the addition of an equal volume of 11.5N HCl and by heating to 105° C. for 5 hours. As higher concentrations of salt have been found to interfere with hexosamine determination, material isolated with magnesium chloride was precipitated by addition of 3 ml. water and 1 ml. 1% CPC to the 1 ml. volume of column eluate. The precipitate produced was recovered by centrifugation and hydrolyzed in 6N HCl as above. Hexosamine was determined on the hydrolysates by the Blix modification of the Elson and Morgan procedure.[5]

Results

No hexosamine could be demonstrated in any of the fractions eluted with 1% CPC. This was expected as mucopolysaccharides had been precipitated previously by the addition of CPC and, thus, CPC soluble material would be lost in the supernatant. However, together with glycopeptides, the only connective tissue mucopolysaccharide normally expected to occur in this fraction is keratan sulfate and we have shown this to be absent from wound granulation tissue.[4] No hexosamine was seen in the 2M magnesium chloride eluate. On the

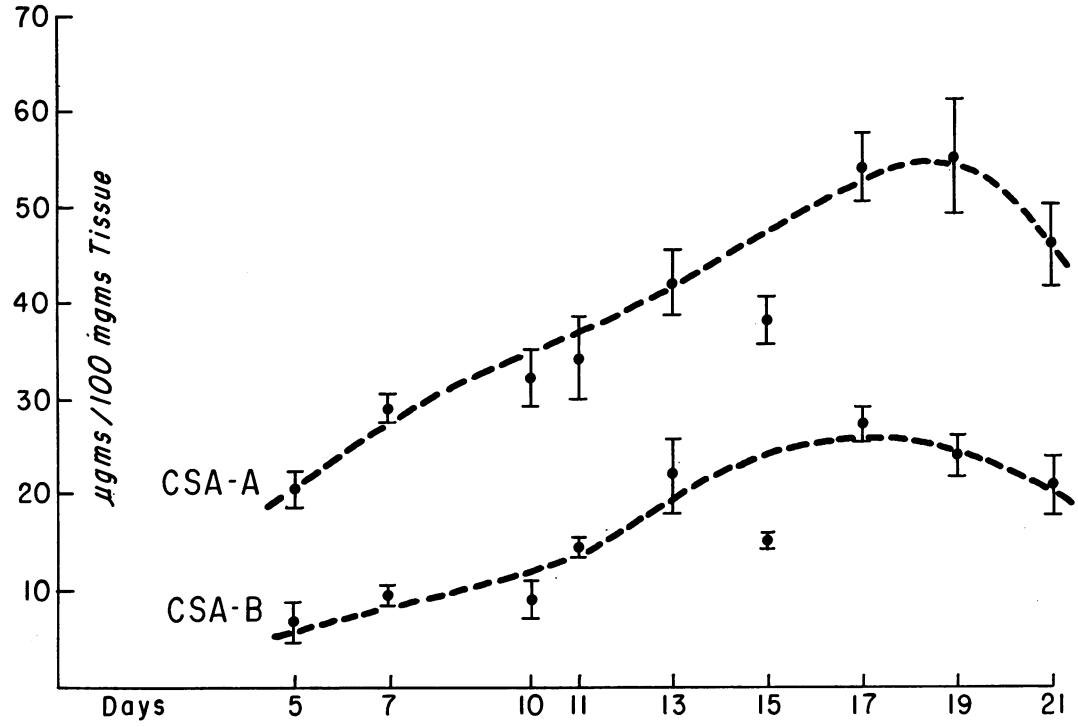

FIG. 1. Chondroitin-4-Sulfate (CSA-A) and Dermatan Sulfate (CSA-B) content of skin wound granulation tissue. (Results expressed as mean ±1 S.D.)

basis of previous work[4] material isolated with 0.75M magnesium chloride in acetic acid will be referred to as chondroitin-4-sulfate (CSA-A) and the material isolated with 0.75M magnesium chloride in water will be referred to as dermatan sulfate (CSA-B). Subject to certain reservations, the material isolated with 0.3N sodium chloride will be referred to as hyaluronic acid (HA). The changes seen in these various components are shown in Figures 1 and 2 and in Table 1.

A progressive rise is seen in total MPS content which is largely due to increasing concentrations of CSA-A and CSA-B. A small initial fall in the HA fraction is followed by relatively little change until 19 days. The percentage composition shows a dramatic but largely artificial fall in the HA fraction due to large increases in the

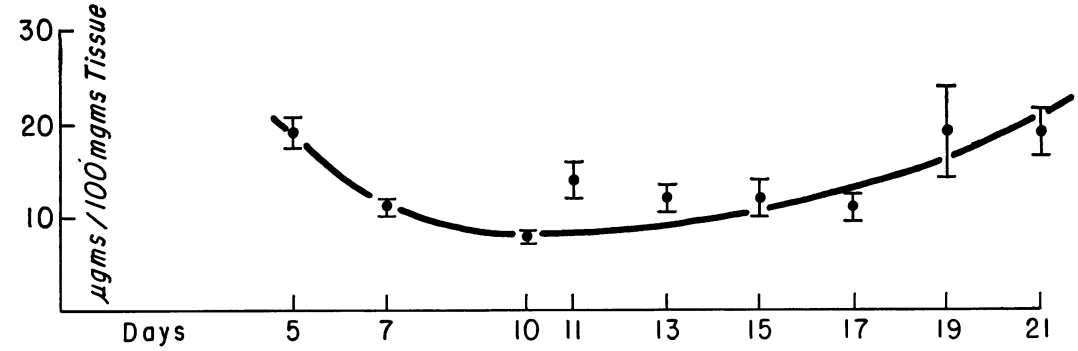

FIG. 2. Content of Hyaluronic Acid (0.3M NaCl fraction) in skin wound granulation tissue. (Results expressed as mean ±1 S.D.)

TABLE 1. *Mucopolysaccharide Content of Wound Granulation Tissue*

Time after Wounding (days)	Total MPS Content (μg./100 mg. tissue)	0.3M NaCl Fraction (HA)		0.75M $MgCl_2$ in Acid Fraction (CSA-A)		0.75M $MgCl_2$ Neutral Fraction (CSA-B)	
		(μg./100 mg. tissue)	(% of total)	(μg./100 mg. tissue)	(% of total)	(μg./100 mg. tissue)	(% of total)
5	47.10	18.78 ± 1.54	39.8	21.33 ± 1.74	45.2	6.99 ± 2.11	14.8
7	50.27	11.24 ± 1.13	22.3	29.44 ± 2.77	58.5	9.59 ± 0.73	19.0
10	49.85	8.15 ± 0.58	16.3	32.77 ± 2.77	65.7	8.93 ± 2.08	17.9
11	62.47	13.66 ± 2.51	21.8	34.42 ± 4.25	55.0	14.39 ± 0.78	23.0
13	76.19	11.80 ± 1.49	15.4	42.14 ± 3.47	55.3	22.25 ± 4.13	29.2
15	64.97	11.73 ± 2.09	18.0	38.18 ± 2.57	58.7	15.06 ± 0.58	23.1
17	91.91	10.66 ± 1.79	11.5	53.93 ± 3.53	58.6	26.91 ± 1.76	29.2
19	98.73	19.05 ± 4.76	19.2	55.14 ± 6.91	55.8	24.54 ± 2.05	24.8
21	86.40	18.96 ± 2.47	21.9	45.94 ± 4.94	53.1	21.51 ± 3.07	24.8

Results are presented as the mean of eight separation procedures plus or minus one standard deviation.
MPS content was calculated by multiplying the measured hexosamine values by appropriate factors derived from the well established structures of the MPS. Thus, HA contains 41% hexosamine and CSA contains 34.6% hexosamine w/w.

other two components. Similar findings were reported by White *et al.*[20] in studies of an analogous tissue, i.e., that which forms inside subcutaneously implanted wire mesh cylinders; the so-called Schilling Tubes. These authors carried out electrophoretic separations of MPS from the tissue. On the basis of staining density of the electrophoresis strips, they concluded that there was a progressive fall in hyaluronic acid content from 7 to 28 days and an increase in mixed CSA from 14 to 35 days. The separation and quantitation of MPS by the use of electrophoresis is less than ideal and it is believed that more recently developed technics presented here permit more effective separations to be carried out.

Discussion

Identity of Mucopolysaccharides. Fractionation procedures used are based upon the original observations of Scott[19] that cetyl pyridinium chloride (CPC), a quaternary nitrogen detergent, will form water-insoluble complexes with mucopolysaccharides (MPS) and other polyanions. Complexes thus formed are soluble, in e.g., sodium chloride solutions to varying degrees dependent on their charge density (a combination of molecular weight and degree of, e.g., sulfation).

By precipitating a mixture of MPS on the surface of a cellulose column previously charged with CPC and eluting with salt solutions of empirically determined concentrations, the various MPS can be separated.

As mentioned above, concentration of salt required to elute a particular MPS (the so-called Critical Salt Concentration or CSC) is dependent upon the molecular weight of the MPS and, indeed, individual MPS can be separated into a range of molecular weights by elution from a column with slightly differing concentrations of salt[15] or by the use of gradient elution technics (Bentley, unpublished observation).

It follows that CSC cannot be used alone as a criterion for identification of an MPS soluble in that concentration, as the average molecular weight of, e.g., chondroitin sulfate may differ from organ to organ and species to species.

In the present investigation, this objection is minimized as the MPS of the same tissue, eluted with the identical salt concentrations used here have previously been rigorously characterized by a variety of methods.[4]

MPS eluted with 0.3M NaCl probably consists of hyaluronic acid contaminated with low molecular weight chondroitin sulfate since both glucosamine and galactosamine were present in this fraction of 10-day wound granulation tissue.[4] Earlier time points might be expected to contain relatively more low molecular weight CSA

if this material is synthesized by chain lengthening. Interpretation of changes seen in the 0.3M NaCl fraction are difficult to make.

Chondroitin Sulfate Synthesis. The progressive increase seen in the concentration of the CSA-A and CSA-B follows a pattern similar to that previously seen for collagen [8, 12] with a maximum slope at 7 to 10 days followed by a plateau at about 17 days. This suggests that massive early production of chondroitin sulfate is not a prerequisite for collagen biosynthesis. On the other hand, the rise of sulfated MPS (Fig. 1) coupled with a small but significant fall (Fig. 2) of the 0.3M NaCl fraction which contains largely hyaluronic acid is in agreement with the theory of fibroblastic control put forward by Balazs and Holmgren [3] and by Campani *et al.*[6] These authors offered evidence which suggest that prior to 4 days, wound granulation tissue contained predominantly non-sulfated polysaccharides which were replaced by sulfated polysaccharides as healing progressed. Predominance of the former in early wounds was felt to mediate for rapid proliferation of fibroblasts whereas differentiation and collagen production were stimulated by the latter.

This suggested mechanism, although apparently supported by the sequence of events, becomes difficult to understand when one considers the source of the MPS in question, for convincing *in vitro* demonstrations have shown that MPS and collagen are synthesized by the same cells.[10, 16, 17] In fact, the production of MPS and collagen may be coincidental and completely unrelated activities of granulation tissue fibroblasts.

Summary

The mucopolysaccharides of open skin wound granulation tissue were separated and quantitated at intervals during the course of healing.

Chondroitin-4-sulfate and dermatan sulfate were seen to increase progressively in concentration from the fifth to the seventeenth day, while the hyaluronic acid-containing fraction remained relatively constant following an initial fall from the fifth to tenth day.

It is concluded that the maintenance of a high concentration of mucopolysaccharides in open wound granulation tissue is not a feature of the first few days of the healing process.

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References

1. Antonopoulos, C. A., Gardell, S., Szirmai, J. A. and De Tyssonsk, E. R.: Determination of Glycosaminoglycans (Mucopolysaccharides) from Tissues on the Microgram Scale. Biochem. Biophys. Acta, **83**:1, 1964.
2. Antonopoulos, C. A., Gardell, S. and Hamnstrom, B.: Separation of the Glycosaminoglycans (Mucopolysaccharides) from Aorta by a Column Procedure Using Quaternary Ammonium Compounds. J. Atheroscler. Res., **5**:9, 1965.
3. Balazs, A. and Holmgren, H. J.: The Basic Dye Uptake and the Presence of a Growth Inhibiting Substance in the Healing Tissue of Skin Wounds. Exp. Cell. Res., **1**:206, 1950.
4. Bentley, J. P.: The Chondroitin Sulfates of Healing Skin Wounds. Acta Chem. Scand., **19**:2235, 1965.
5. Blix, G.: The Determination of Hexosamine According to Elson and Morgan. Acta. Chem. Scand., **2**:467, 1948.
6. Campani, M., Zonta, A. and Ugazio, G.: Ricerche sul "tessuto di riparazione" delle ferite cutanee. Proc. Simposio della Societa Italiana E della Societa Tedesca Di Patologia, Milano 6–7 October, 1959.
7. DeVito, R. V.: Healing of Wounds. Surg. Clin. N. Amer., **45**:441, 1965.
8. Dunphy, J. E. and Udupa, K. N.: Chemical and Histochemical Sequences in Normal Healing of Wounds. New Eng. J. Med., **253**:847, 1955.
9. Edwards, L. C., Pernokas, L. N. and Dunphy, J. E.: The Use of a Plastic Sponge to Sample Regenerating Tissue in Healing Wounds. Surg. Gynec. Obstet., **105**:303, 1957.
10. Green, H. and Goldberg, B.: Kinetics of Collagen Synthesis by Established Mammalian Cell Lines. Nature, **200**:1097, 1963.
11. Hale, C. W.: Histochemical Demonstration of Acid Polysaccharides in Animal Tissues. Nature, **157**:802, 1946.
12. Hosoda, Y.: Studies on Granulation Tissue. I. Soluble Collagen in Wound Healing. II.

Fibrogenesis and Nucleic Acids. Keio J. Med., **9**:261, 1960.

13. Hotchkiss, R. D.: Microchemical Reaction Resulting in Staining of Polysaccharide Structures in Fixed Tissue Preparations. Arch. Biochem., **16**:131, 1948.
14. Jackson, D. S., Flickinger, D. B. and Dunphy, J. E.: Biochemical Studies of Connective Tissue Repair. Ann. N. Y. Acad. Sci., **86**: 943, 1960.
15. Laurent, T. C. and Scott, J. E.: Molecular Weight Fractionation of Polyanions by Cetylpyridinium Chloride in Salt Solutions. Nature, **202**:661, 1964.
16. Morris, C. C. and Godman, G. C.: Production of Acid Mucopolysaccharides by Fibroblasts in Cell Cultures. Nature, **188**:407, 1960.
17. Prockop, D. J., Pettingill, O. and Holtzer, H.: Incorporation of Sulfate and the Synthesis of Collagen by Cultures of Embryonic Chondrocytes. Biochim. et Biophys. Acta, **83**:189, 1964.
18. Rinehart, J. F. and Abul-Haj, S. K.: Improved Method for Histological Demonstration of Acid Mucopolysaccharides in Tissues. Arch. Path., **52**:189, 1951.
19. Scott, J. E.: Aliphatic Ammonium Salts in the Assay of Acidic Polysaccharides from Tissues. *In* Glick, D. (ed.), Methods of Biochemical Analysis, Vol. 8. New York, Interscience, 1960, p. 145.
20. White, B. N., Shetlar, M. R. and Schilling, J. A.: The Glycoproteins and their Relationship to the Healing of Wounds. Ann. N. Y. Acad. Sci., **94**:297, 1961.